Contents lists available at SciVerse ScienceDirect

Food and Bioproducts Processing

journal homepage: www.elsevier.com/locate/fbp

Kinetics, aggregation behavior and optimization of the fractionation of whey protein isolate with hydrochloric acid☆

Laetitia M. Bonnaillie, Peggy M. Tomasula*

Agricultural Research Service, U.S. Department of Agriculture, Eastern Regional Research Center, Dairy & Functional Foods Research Unit, 600 East Mermaid Lane, Wyndmoor, PA 19038, United States

A B S T R A C T

Concentrated WPI solutions (10%, w/w) containing approximately 50% beta-lactoglobulin (β-LG) and 26% alpha-lactalbumin (α-LA) were fractionated with HCl at acidic pH and moderate temperature to obtain enriched α-LA and β-LG fractions. Aggregation behavior and kinetics of protein precipitation and aggregate formation were analyzed as a function of four process parameters: pH (3.0–5.5), temperature (50–70 °C), reaction time (0–180 min) and protein concentration (10–29%). The precipitation and aggregation of α-LA appeared rate-limited, with a logarithmic dependence of time and possible bimodal nucleation rate, and varied considerably with pH and temperature. Aggregates as large as ~300 μm were noted after 120 min at pH 4, 60 °C. Processing parameters were optimized to obtain both a high aggregate yield and optimal composition of the aggregate fraction. The optimally enriched solid and liquid fractions contained 58% α-LA and 76% β-LG, respectively, with 99% and 74% recovery ratios. Over the pH range studied, β-LG aggregation was found negligible at 60 °C and β-LG recovery in the aggregates attributed to liquid holding. Increasing WPI concentration accelerated α-LA aggregation, demonstrating a concentration-dependent aggregation mechanism, and reduced aggregate purity. Enriched whey protein fractions are valuable health-enhancing food ingredients.

Published by Elsevier B.V. on behalf of The Institution of Chemical Engineers.

Keywords: Whey proteins; Aggregation; Fractionation; Alpha-lactalbumin; Beta-lactoglobulin; Kinetics; Hydrochloric acid

1. Introduction

The main byproduct of cheese manufacturing is sweet whey. Just over 50% of cheese whey produced in the U.S. is concentrated through ultrafiltration or ion-exchange to manufacture whey protein concentrates (WPC) and whey protein isolates (WPI) (Bonnaillie and Tomasula, 2008). WPC contain from 34 to 85 wt.% protein, while WPI contain less lactose and fat, and more than 90 wt.% protein. β-Lactoglobulin (β-LG) usually constitutes more than half of the total composition of WPI, whose functional properties approximate those of β-LG.

Fractionation of the whey proteins can emphasize the functional and nutritional properties of the individual proteins. Reviews of fractionation techniques that have been designed to separate β-LG and α-LA from the other whey proteins using clarified whey, WPC or WPI solutions can be found in Bonnaillie and Tomasula (2008) and El-Sayed and Chase (2011). Techniques to fractionate the whey proteins include the selective aggregation of either β-LG or α-LA *via* heat denaturation, with or without pH adjustment to exploit the different isoelectric points of α-LA (~4.4) and β-LG (5.2) (McKenzie, 1971). While β-LG precipitates rapidly and selectively at high temperature (70–120 °C) and pH near neutral (pH-8) (Kiesner et al., 2000; Plock et al., 1998; Wang et al., 2006), α-LA precipitates and aggregates better at acidic pH (3.5–5.5) and moderate temperature (50–65 °C) with long reaction times, usually accompanied by the precipitation of bovine serum albumin (BSA), immunoglobulins (Ig) and lactoferrin (Lf), while β-LG and casein-macropeptide (CMP) remain soluble (Bramaud et al., 1997b; Fernandez et al., 2011; Outinen et al., 1996; Pearce et al., 1991a). At neutral pH, α-LA contains 1 mol of calcium per mol of protein, stabilizing the folded protein *via* internal linkages (Kilara and Vaghela, 2004). With the addition of acid such as hydrochloric acid (HCl) to heated

☆ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* *Corresponding author*. Tel.: +1 215 836 3782; fax: +1 215 233 6470.
E-mail addresses: laetitia.bonnaillie@ars.usda.gov (L.M. Bonnaillie), peggy.tomasula@ars.usda.gov (P.M. Tomasula).

Received 16 March 2011; Received in revised form 19 December 2011; Accepted 11 January 2012
0960-3085/$ – see front matter Published by Elsevier B.V. on behalf of The Institution of Chemical Engineers.
doi:10.1016/j.fbp.2012.01.002

whey, WPC or WPI solutions, calcium ions within α-LA dissolve around pH 3.8–4.2 and α-La unfolds and aggregates (Bernal and Jelen, 1984; Bramaud et al., 1997a; Pearce, 1983; Rialland and Barbier, 1988; Wu, 2003), forming molten globules (Permyakov and Berliner, 2000). The α-LA precipitate is extracted by centrifugation. After separation of the two protein fractions, neutralizing the acidic pH introduces salts in the products. Because removal of these salts generates extra processing steps and cost, supercritical CO_2 was used to acidify WPC solutions by formation of carbonic acid. After depressurization, CO_2 was released and pH returned to 6–6.3 (Tomasula and Parris, 1999; Tomasula et al., 1998).

At neutral pH, the kinetics of heat-induced denaturation of individual whey proteins were studied at various temperatures, protein concentrations and mineral contents (Dannenberg and Kessler, 1988; Kessler and Beyer, 1991). In dilute conditions at high temperature (≥80 °C), the rates of thermal denaturation of α-LA and β-LG had an order of 1 (Tolkach et al., 2005) and 1.5–2, respectively (Roefs and de Kruif, 1994; Verheul et al., 1998). The kinetics of formation and the morphology of salt- or thermally-induced WPC or WPI aggregates near neutral pH and at high temperature were also characterized (Havea et al., 2002; Schmitt et al., 2007; Wu et al., 2005).On the other hand, acidic pH considerably affects the denaturation and aggregation rates of both α-LA and β-LG (Tolkach and Kulozik, 2005), and limited data is available on the quantitative effects of pH on precipitation and aggregation kinetics. At 80 °C, the thermal denaturation rate of β-LG decreases considerably from pH 6.7 to 4.5 (Spiegel and Huss, 2002), enabling the selective recovery of α-LA aggregates around pH 4.5. However, an analysis of pH effects on the precipitation kinetics and aggregation behaviors of α-LA and β-LG at acidic pH and lower temperatures (50–70 °C) is lacking.

The present work aims to extend Pearce's studies (1983; 1987; 1991) of the precipitation of α-LA with HCl at moderate temperatures with a study of the kinetics and behavior of the α-LA aggregation reaction as a function of time, temperature, pH and protein concentration, in order to optimize the separation of α-LA-enriched aggregates from the β-LG-enriched soluble fraction and maximize both the yield and purity of the products, for commercial scale-up purposes. We used concentrated WPI as a starting material to lower the amount of impurities in the protein solution (lactose, fat, salts) compared to WPC or dilute whey, in order to study the individual rates of precipitation of α-LA and β-LG and optimize the reaction parameters to maximize α-LA precipitation while minimizing that of β-LG. Post-processing steps that alter the protein fractions (*e.g.*, washing) were not used, to quantify aggregate yields and protein recoveries as a function of sole reaction conditions.

Results from this study help understand and optimize new or existing acid-induced whey protein fractionation processes that use HCl or other acids, such as CO_2 (Tomasula and Yee, 2001), and increase the yield and purity of the protein products.

2. Materials and methods

2.1. Materials

Spray-dried WPI from cheese whey, Provon 190, was purchased from Glanbia Nutritionals Inc. (Richfield, ID) and contained 90.1% (w/w) protein, with 3.6% moisture, 2.9% ash, and fat and lactose. The protein composition measured with gel electrophoresis (SDS-PAGE, 5 replicates) was: 59.3% (w/w) β-LG, 31% α-LA, 3.3% caseins, 3.1% Ig, 2.6% BSA and 0.7% Lf. The CMP content was estimated to ∼15% (w/w) using a UV-spec/RP-HPLC/mass-spec combination method (unpublished results) and the protein composition of WPI was corrected to: 50.5% β-LG, 26.3% α-LA, 15% CMP and 8.2% other proteins.

A 4 N hydrochloric acid (HCl) solution (Sigma–Aldrich, St. Louis, MO) was used to adjust the pH of WPI solutions as desired. All WPI solutions were prepared with de-ionized water produced with a Milli-Q Synthesis water purification system (Millipore, Billerica, MA).

2.2. Study of aggregation kinetics vs. pH and time

Solutions containing 10% (w/w) WPI (*i.e.*, 9.0% protein) in de-ionized water were turbulently stirred in an Erlenmeyer flask at room temperature until dissolved. The flask was then placed in a water bath (temperature stability ± 0.05 °C) heated to 50, 55, 57.5, 60, 65 or 70 °C, and stirred quickly with an impeller. After thermal equilibrium (∼10 min), a pre-calculated amount of HCl was injected to lower the pH of the WPI solution to the working pH of interest (from 5.5 to 3.8) and initiate the aggregation of α-LA. Kinetic samples (5 mL) were extracted with a pipette before the addition of HCl at $t_0 = 0$, and then at times t_i and transferred to pre-weighed, 5 mL glass centrifuge tubes held in an ice-bath to quench the aggregation reaction. Samples were also collected in 2.5 mL sealed plastic tubes for microscopic imaging purposes. Kinetic experiments were run for up to 300 min.

2.3. pH adjustment

Due to the buffering properties of whey proteins, we first calibrated the pH of the WPI solutions as a function of the volume of 4 N HCl added. Solutions with 10% (w/w) WPI were prepared and heated to 60 °C in a digital water bath and stirred rapidly. Solution were yellow and translucent at the initial pH of 6.22. Every 10 min, small HCl aliquots were added to reduce the pH slightly and the pH, opacity and viscosity of the WPI solution were noted. A linear relationship between pH and the volume of HCl added was noted between pH 5.0 and 3.0: $pH = -81.4\,V_{HCl} + 5.62$, where V_{HCl} is the volume of 4 N HCl added to a 10% WPI solution, in mL/mL. This formula was used in all kinetic experiments to calculate the volume of HCl to add at t_0. The pH of the WPI solutions was measured both before and after each fractionation experiment.

2.4. Microscopic study of aggregate formation

Kinetic samples placed in small plastic tubes were fixed with the addition of 10% (v/v) of a 25% glutaraldehyde solution at room temperature, then left to solidify overnight under refrigeration for microscopic imaging. Solidified samples were thinly sliced in their center with a razor blade, and protein population-density on the slice was analyzed with confocal fluorescence microscopy using fluorescent functional groups such as tryptophan (Vivian and Callis, 2001).

2.5. Separation and quantification of the protein fractions

Kinetic samples in glass tubes were covered and stored upright overnight in the refrigerator. After ∼16 h, the sedimentation

profile (opacity as a function of vertical depth) of the aggregate particles inside each tube was examined to derive kinetic information on particle size evolution and yield *vs.* reaction time and pH. The solid and soluble protein fractions were then separated using a table-top Sorvall® EconoSpin centrifuge with bucket holders, for 60 min at room temperature and maximum speed (3600 rpm, average acceleration = 2000 × *g*). The supernatant was collected into glass vials and labeled 'beta' fraction, or the protein fraction enriched with β-LG. The aggregate fraction was lyophilized and labeled 'alpha' fraction, or the protein fraction enriched with α-LA. Both protein fractions were quantified by differential weighing: before centrifugation; after removal of the supernatant; after lyophilization. Post-treatments such as washing and ultrafiltration were not applied to minimize material losses and weighing errors.

2.6. *Composition of protein fractions*

Gel electrophoresis samples of all fractions were prepared with 7 wt.% protein in a 2.5% SDS solution neutralized with sodium hydroxide 0.1 N. SDS-PAGE gels were run on a Phast System (Pharmacia, Piscataway, NJ) using 20% acrylamide homogeneous gels with 8 lanes. A low-molecular weight marker (Bio-Rad, Hercules, CA) was used on one of the gel lanes. Gels were stained with Coomassie brilliant blue dye at room temperature for 20 min and de-stained overnight. Gels were analyzed with ImageQuaNT™ software (Molecular Dynamics Inc., Sunnyvale, CA). The quantitative protein composition of each sample was determined from the relative band sizes ('band %') and integrated color-density profiles ('volume') of each band as calculated with the ImageQuaNT™ software. Data analyses of each sample were performed in duplicate.

3. Results and discussion

3.1. *Kinetics of aggregate growth*

Under acidic pH at 60 °C, α-LA and most of the minor whey proteins precipitated, while CMP and most of the β-LG remained in solution. The size, rate of formation, yield and composition of the aggregates were functions of pH; time, *t*; temperature, *T*; and protein concentration, *C*.

3.1.1. *pH range determination*

The pH of a highly concentrated WPI solution (28.5%, w/w) was adjusted progressively to determine the ideal pH range of study at 60 °C. Whey proteins aggregated and/or polymerized when acidity increased and caused respective whitening and/or thickening of the WPI solution. From pH 6.2–5.5, no change was observed. Below pH 5.5, whitening and increasing opacity of the WPI solution were noted and attributed to progressive generation of protein particles. A noticeable increase of the solution's viscosity was also observed at low pH. At pH 3.8, some gelling was noted, hypothetically due to the polymerization of β-LG proteins, and a thick gel was fully formed at pH 3.3. Thus, pH 5.5–3.8 was chosen as the optimal pH range to study whey protein aggregation at 60 °C without gelling interference.

3.1.2. *Particle size*

The growth of individual aggregate particles was first evidenced by tracking the evolution of the vertical opacity gradient of each kinetic sample during settling under normal gravity. Immediately after extraction from the reactor, all samples were uniformly white and opaque. During settling, an opacity gradient appeared as particles began descending through the samples, forming a sedimentation front. Larger particles sediment faster than smaller ones because the sedimentation velocity is proportional to the square of diameter according to Stoke's law (Lamb, 1994). At all values of pH, a downward shift of the sedimentation front and increased transparency of the topmost layer of the samples with reaction time (*t*) evidenced an acceleration of particle sedimentation caused by particle growth. At pH 4 and $0 < t < 40$ min, the tranparency of top sample layers after settling increased with reaction time due to the growth of fine particles and consequent sedimentation rate-increase; for $t > 40$ min, top sample layers remained opaque after overnight settling. The presence of suspended fines suggested either a bimodal aggregation rate of the whey proteins, or increased mixture viscosity. Particle size analysis of a sample extracted at pH 4 and $t = 224$ min with a Model 780 AccuSizer particle size analyzer (Agilent Technologies, Santa Clara, CA) in de-ionized water, showed that ~53% of the particles detected had a 1–2 μm diameter; ~20%, 2–5 μm diameter; and the remaining ~27% followed a bell-shaped distribution between 5 and 50 μm diameter. The number-average diameter was 4.8 μm, volume-average diameter, 22 μm, and polydispersity index, 4.6. The AccuSizer was however not a reliable technology to determine the size distribution of whey protein aggregates immediately after fractionation: partial redissolution of the aggregates in water during analyses shifted the entire distribution toward smaller particle sizes, as evidenced by the number-average diameter of 3.4 μm and polydispersity index of 12.3, after 90 min residence in the AccuSizer.

The kinetics of aggregate formation were observed with confocal fluorescence microscopy. Fig. 1 shows the evolution of the structure of the WPI solution with reaction time at 60 °C and pH 4 (negative image). Shades of grey are a function of local protein density and darker areas represent zones of denser protein population. At $t = 0$, the solution is a mostly homogeneous suspension, with a few small particles of undissolved WPI powder present. After 1 min, the solution loses its homogeneity as proteins begin to flocculate. At $t = 6$ min, aggregated protein areas are observed (darker areas), that grow and become denser and more defined with time, while the homogeneous background (light grey area) recedes. At $t = 99$ min, large aggregates of 200–300 μm diameter can be seen, which is approximately an order of magnitude larger than the largest particles measured with the AccuSizer. A number of very small particles (<10 μm) become visible in the background, in agreement with suspended fines observed during sedimentation studies. Further analyses may determine whether these fines are small α-LA aggregates or aggregates of the sparse minor whey proteins.

3.1.3. *Total aggregate yield*

The total aggregate yield, Y_{agg}, was defined as the ratio of the mass of dry aggregate collected after centrifugation and lyophilization, to the initial mass of WPI powder in a sample:

$$Y_{agg} = \frac{\text{mass dry aggregate}}{(\text{mass sample} \times C)} \quad (1)$$

where *C* is the WPI solution concentration (w/w). At all values of pH, the total aggregate yield increased with time during the aggregation reaction, and was fitted very well with a

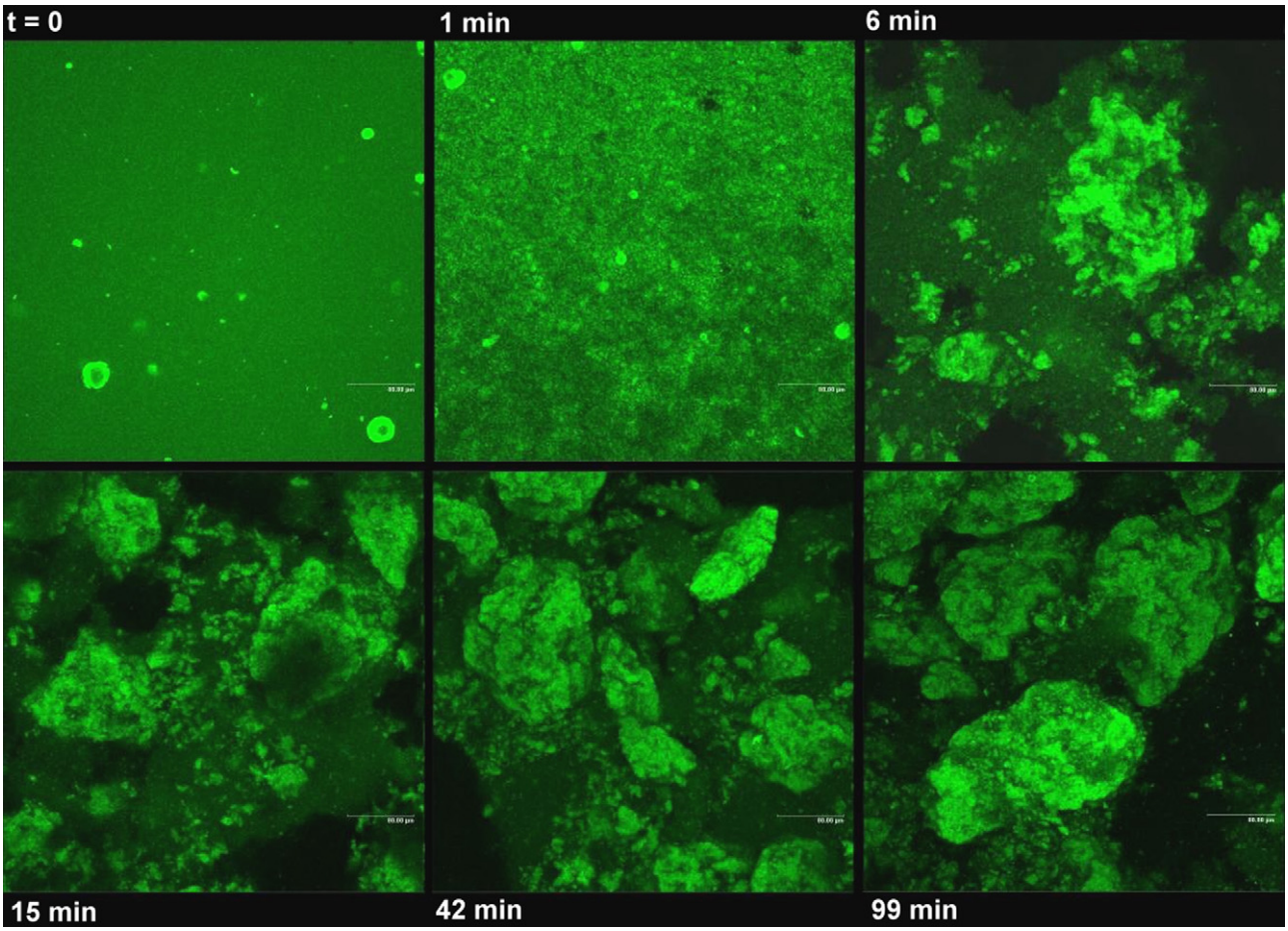

Fig. 1 – Confocal auto-fluorescence microscopic images of 10% WPI solution *vs.* time, during aggregation at 60 °C and pH 4. Negative image: darker areas represent zones of denser protein population. Scale: 80 μm.

logarithmic function of time, *e.g.*, $Y_{agg}(t) = 0.044\ \ln(t) + 0.195$ at pH 4.28, with t in minutes.

3.2. *Fractions compositions and protein recoveries*

3.2.1. *Compositions measured with SDS-PAGE*

In Fig. 2, a SDS-PAGE gels shows the typical evolution of the protein composition of the beta fraction with time, during the aggregation reaction. In each lane, the α-LA band is located at 14,400 g/mol and the β-LG band around 18,000 g/mol, while all the other bands constitute BSA, Lf, Ig, and caseins and casein residues, which were all grouped under the 'minor whey proteins' category because or their small percentages in the starting WPI. Fig. 2 clearly shows the depletion of α-LA from the liquid fraction with time, due to precipitation and aggregation of α-LA. At 60 °C, α-LA precipitation was slow and progressive, and equilibrium was not reached after 224 min at pH 4.06. On the contrary, the minor whey proteins precipitated

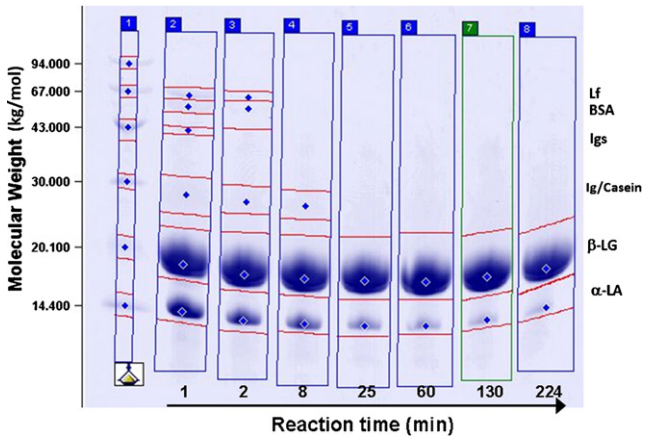

Fig. 2 – Evolution of the protein content of the beta (liquid) fraction *vs.* time, during aggregation at 60 °C and pH 4, as analyzed with SDS-PAGE. Lane 1: low molecular weight marker.

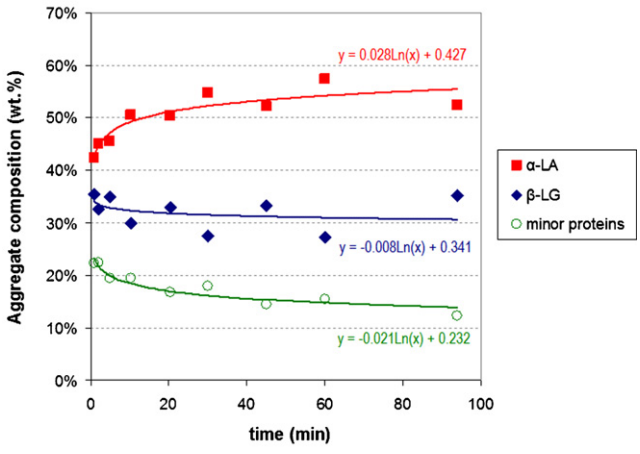

Fig. 3 – Composition of the aggregate fraction *vs.* time during fractionation of 10% WPI solution at 60 °C and pH 4.7, from SDS-PAGE measurements: evolution of α-LA, β-LG, and minor proteins contents.

quickly and the corresponding bands on gels became too faint to identify and measure after ~10 min.

Fig. 3 presents the quantitative evolution of the composition of the aggregate fraction at pH 4.7. Minor proteins appeared to precipitate first and their content in the aggregate fraction was highest at the beginning. As time increased, α-LA precipitated faster than either β-LG or the minor proteins and the proportion of α-LA in the aggregates increased, resulting in relative enrichment of the precipitate with α-LA.

3.2.2. *Protein recoveries: kinetics of α-LA and β-LG aggregation*

Due to high solubility in the range of temperature and pH used, most of the lactose, minerals, and CMP in WPI were assumed to remain in solution during centrifugation and be recovered in the supernatant, together with the low-density fat, while the aggregate fraction consisted mostly of proteins. The recovery yields for α-LA, R_A, and for β-LG, R_B, were calculated as follows:

$$R_A = \frac{\text{amount of α-LA in aggregate}}{\text{initial amount of α-LA}} = \frac{x_A \times Y_{agg}}{x_{A,0}} \quad (2)$$

$$R_B = \frac{\text{amount of β-LG in aggregate}}{\text{initial amount of β-LG}} = \frac{x_B \times Y_{agg}}{x_{B,0}} \quad (3)$$

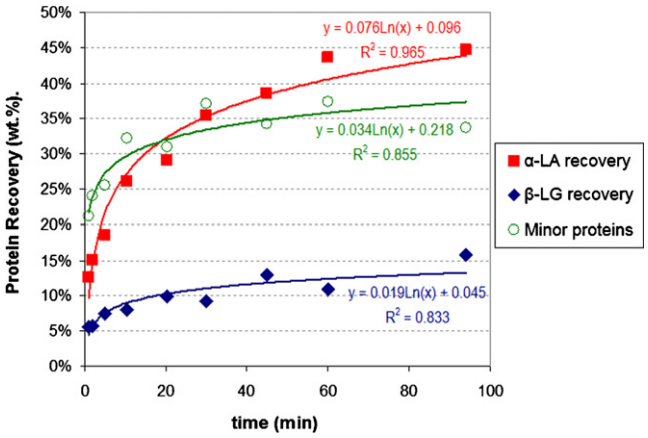

Fig. 4 – Protein recovery yields in the aggregate fraction during fractionation of a 10% WPI solution at 60 °C and pH 4.70.

where $x_{A,0}$ and $x_{B,0}$ are the mass fractions of α-LA and β-LG in the initial WPI, respectively, and x_A and x_B are the mass fractions of α-LA and β-LG in the aggregate fraction measured with SDS-PAGE.

Fig. 4 shows the fast initial precipitation of the minor proteins tapering off after 10 min, followed by continued aggregation of the α-LA proteins with time (up to 45% recovery after 95 min at pH 4.7). The smaller amount of β-LG recovered

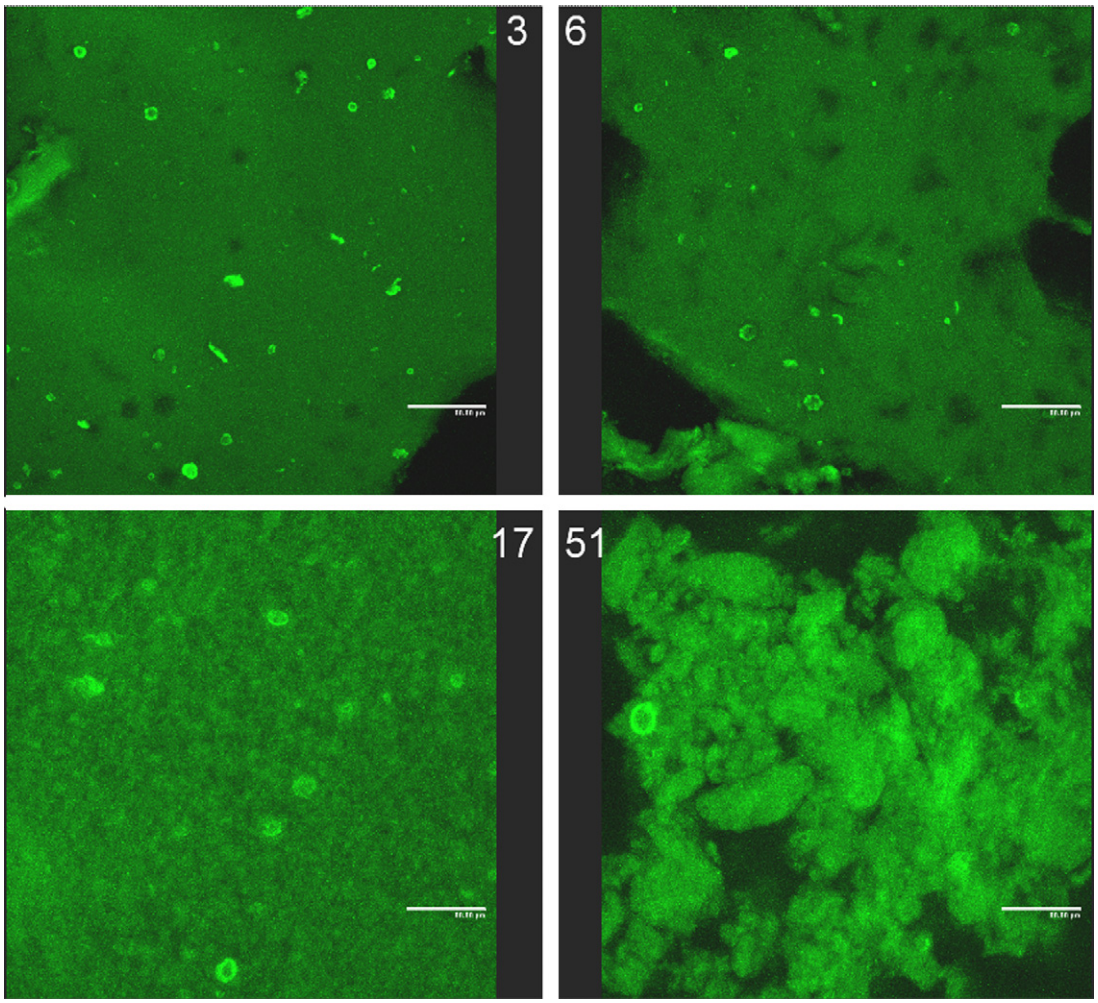

Fig. 5 – Confocal auto-fluorescence microscopic images of a 10% WPI solution during aggregation at pH 3.5 and 60 °C. Samples collected after 3, 6, 17 or 51 min. Negative images: darker areas represent zones of denser protein population. Scale: 80 μm.

in the aggregate fraction may be due in part to entrapment of β-LG proteins within the α-LA and minor whey protein aggregates, and in part to the precipitation of smaller amounts of β-LG, since β-LG is more thermally stable than α-LA at acidic pH (Boye and Alli, 2000). In the entire range of pH studied, both the recovery rates of α-LA and β-LG usually followed a logarithmic profile, with α-LA precipitating much faster than β-LG, which is desirable to produce an aggregate fraction enriched with α-LA and a liquid fraction enriched with β-LG. All data sets were modeled with logarithmic curves prior to further analysis.

3.3. *Effects of pH*

3.3.1. *Kinetics of aggregates formation*

At pH 3.5 and below, WPI solutions were observed to gel almost instantly after the addition of HCl at 60 °C. The opaque solutions, densely populated with aggregates, became too viscous to allow sedimentation of particles during overnight refrigeration, and separation of the two fractions *via* centrifugation was poor. Fig. 5 illustrates the formation of α-LA aggregates within a β-LG-based gel at pH 3.5 and 60 °C: the instantaneous formation of a continuous, homogeneous gel (top-left picture) dramatically increased viscosity throughout the WPI solution, reducing protein mobility. The onset of α-LA flocculation was delayed compared to pH 4.06 (between 6 and 17 min, *vs.* 1 min in Fig. 1), then numerous dense aggregates larger than 100 μm eventually formed. At pH 3.8 and above, sedimentation profiles under normal gravity suggested a reduction in the amount of particles formed (*i.e.*, reduced opacity) with increasing pH, as well as a decrease in average particle size as evidenced by reduced sedimentation rates. In addition, more pronounced sedimentation gradients through the samples suggested a broadening of particle size distribution with increasing pH and time, and the presence of a large population of fines at pH 4.7 and higher.

3.3.2. *Aggregate yield*

The total aggregate yield at 60 °C and its evolution with time between 1 and 120 min in the range of pH studied are presented in Fig. 6. Below pH 3.8, gelling rendered separation of the aggregate fraction from the gel fraction by centrifugation almost impossible.

Aggregate yield increased with decreasing pH, and the maximum yield was obtained around pH 3.9–4.0, which is considerably lower than α-LA's isoelectric point of 4.4, as Bramaud et al. (1997a) also found while treating dilute WPC with HCl. Aggregate yields increased significantly with reaction time, indicating slow protein aggregation kinetics (equilibrium was not reached after 3 h), except around pH 3.8–3.9, where the aggregation reaction accelerated and became almost instantaneous. This is important for commercial processes where production rates are economically critical.

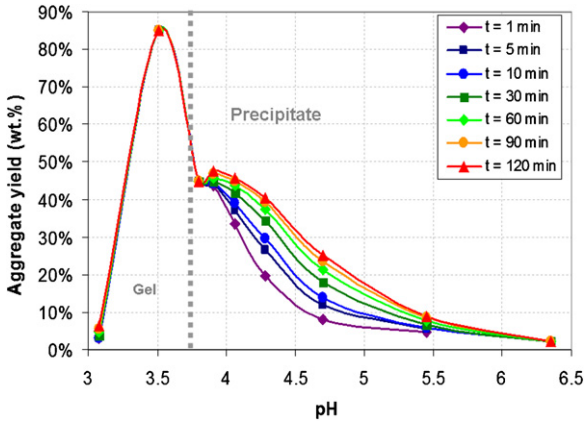

Fig. 6 – Total aggregate yield after centrifugation as a function of pH and time during aggregation of 10% WPI solutions at 60 °C.

3.3.3. *Protein recoveries*

Logarithmic models of kinetic data at each pH value were used to build Fig. 7, featuring the recovery rates (Fig. 7A) and compositions (Fig. 7B) of α-LA and β-LG in the aggregate fraction as a function of time and pH during the aggregation reaction. Recovery of α-LA in the aggregate fraction increased logarithmically with time and was maximized at pH 4, reaching ~99% recovery after 120 min. The recovery rate of β-LG in the aggregate fraction increased with time and decreasing pH above pH 4.1, and became approximately constant at pH 3.8–4.0 after reaching its maximum value of ~30% within 2 min. Because α-LA continued to precipitate with time at pH 3.9–4.0, the proportion of α-LA in the alpha fraction was maximized at pH 4, with up to 62% α-LA after 120 min at pH 4.06. With more dilute (2%) WPC solutions, Bramaud et al. (1997a) similarly found that the aggregation of α-LA at 60 °C was maximized around pH

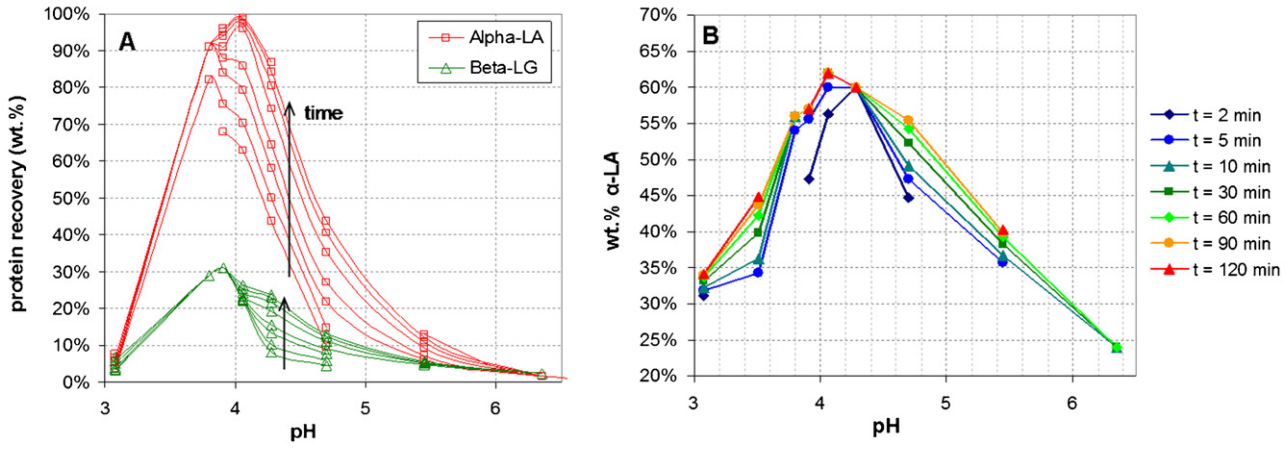

Fig. 7 – (A) Extents of recovery of α-LA and β-LG proteins in the alpha fraction as a function of time (1–120 min) and pH during aggregation at 60 °C. (B) Composition of the aggregate fraction *vs.* pH and time, as measured with SDS-PAGE.

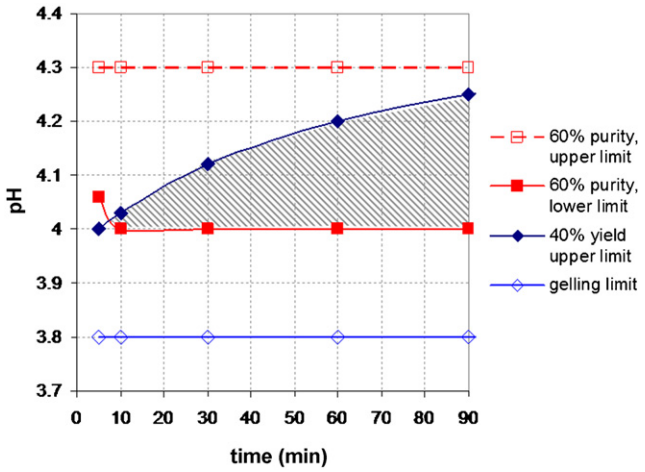

Fig. 8 – Optimization of reaction time and pH during the fractionation of 10% WPI solutions with HCl: the shaded operating area produces an aggregate yield ≥40%, with α-LA content ≥60% (w/w).

3.8–3.9, with ~85% α-LA and almost no β-LG recovered in the aggregate after 120 min.

3.3.4. *Optimization of pH and reaction time at 60 °C*

The total aggregate yield of the process was maximized between pH 3.9 and 4.0 ($Y_{agg} \geq 45\%$) and increased logarithmically with time. With reaction times greater than 1 h, a pH range of 3.8–4.2 produced yields $Y_{agg} \geq 40\%$ (w/w). The proportion of α-LA in the aggregate was maximized at pH 4.0 and α-LA contents increased logarithmically with time, from 55% after 2 min to 62% after 120 min. Lines of constant yield and constant composition defined from Figs. 6 and 7 were used to build Fig. 8, representing the operating pH and time ranges (shaded area) to produce at least 40% precipitate containing at least 60% α-LA from 10% WPI solutions at 60 °C. According to Fig. 8, at least 10 min are needed to obtain both a high process efficiency and a high alpha fraction purity at pH 4.0. The optimal pH range widens with longer reaction times: *e.g.*, after 60 min reaction, the working pH range becomes 4.0–4.2.

3.3.5. *Composition of the beta fraction at pH 4*

At the optimal pH and reaction time defined above (pH 4, $t \geq 10$ min) the recovery of α-LA and the ratio R_A/R_B in the alpha fraction were both maximized, thereby also optimizing the purity of the beta fraction. The β-LG content measured with SDS-PAGE (Fig. 2) was 98% (w/w) after 120 min, corrected to 75% β-LG, 23.5% CMP and 1.5% α-LA after measuring CMP with reverse-phase high-performance liquid chromatography (RP-HPLC).

3.4. *Effects of temperature at pH 4*

3.4.1. *Rates of aggregate formation and growth*

The effects of temperature on the kinetics of aggregate formation and growth, and individual protein precipitation were examined at pH 4 and at 50, 55, 57.5, 65 and 70 °C. Sedimentation profiles evidenced a strong influence of temperature on the formation and growth of α-LA aggregates with time. Rates appeared possibly bimodal at all temperatures and individual particles sizes, growth rate, and total amount of aggregate varied greatly with temperature. At 50 and 55 °C, fine particles began forming at 1 min. The sedimentation rate in samples increased slowly with reaction time as small particles combined to form a growing amount of aggregates large enough to settle overnight. At 20–25 min, the topmost layer of liquid in the samples became clear and free of fines. A population of fines reappeared at 60 min due to bimodal aggregation kinetics or to a rise in solution viscosity from partial gelling of β-LG proteins. At 50 °C, the particle population formed during the first 90 min of reaction was too fine to sediment well during centrifugation; this can be remedied with higher centrifugation speeds. Between 57.5 °C and 65 °C, the rate of aggregation of fine particles into larger ones accelerated with temperature, as evidenced by increased sedimentation rates, faster growth of the sediment fraction with reaction time, and earlier disappearance of fines from the supernatant phase (after 10 min at 57.5 °C; 4 min at 60 °C; and 2 min at 65 °C). At all temperatures, fines reappeared after approximately 1 h.

At 70 °C, a large amount of aggregates formed after 40 min at pH 6.35, prior to the addition of HCl, because the denaturation temperature of α-LA of 67 °C was exceeded (Ju et al., 1999). We also noted a rise in viscosity sufficient to keep these aggregates suspended in the solution. The addition of HCl to pH 4 triggered no visible change in the amount of aggregates or the sedimentation profiles after up to 120 min reaction.

Fig. 9 shows the effect of temperature on aggregate formation at pH 4, using confocal auto-fluorescence microscopy. Compared to 60 °C, aggregate formation slowed considerably at 50 °C and accelerated greatly at 70 °C. After 2 min at 70 °C, the entire aggregate population appeared formed and no evolution in either size or amount of particles was visible with reaction times up to 115 min. On the other hand, protein aggregation was slower and progressive at 50 °C, a large number of nuclei but almost no aggregates being present after 6 min, followed by the appearance of a population of small particles that appeared to grow in both size and number with reaction time. Increasing the fractionation temperature from 50 to 70 °C seemed to significantly increase both the rates of aggregate nucleation and aggregate growth at pH 4. Final aggregate sizes seemed largest at 60 °C, which is of interest to optimize the separation of the fractions since larger aggregates settle better during centrifugation.

3.4.2. *Proteins recoveries and aggregate composition*

Fig. 10 presents the effects of time at pH 4 between 50 and 70 °C on the total aggregate yield, Y_{agg} (Fig. 10A), recovery rates of α-LA and β-LG in the alpha fraction (Fig. 10B and D), and aggregate composition after 120 min (Fig. 10C). Y_{agg} increased almost linearly with temperature (from 40% after 120 min at 50 °C, to 60% at 70 °C) and followed a logarithmic function of time. At 70 °C, Y_{agg} reached 42% *before* the addition of HCl due to the denaturation of α-LA above 67 °C.

The recovery rate of α-LA in the alpha fraction increased approximately linearly with temperature from 50 to 60 °C, and followed a logarithmic function of time at all temperatures during the first 60 min. At 60 °C, α-LA recovery reached a maximum then remained approximately constant between 60 and 70 °C, increasing with time only (Fig. 10B). α-LA recoveries greater than 90% were reached for $t \geq$ ~60 min on the whole range $55 < T < 70$ °C, and was maximized (95–100%) for $t \geq 120$ min.

On the other hand, the precipitation behavior of β-LG as a function of temperature differed greatly from that of α-LA. The rate of precipitation of β-LG plateaued between 50 and 60 °C, with little effect of time, before increasing sharply between 65 and 70 °C (Fig. 10D). Linear fits of these two trends suggest an approximate denaturation temperature of 62.5 °C

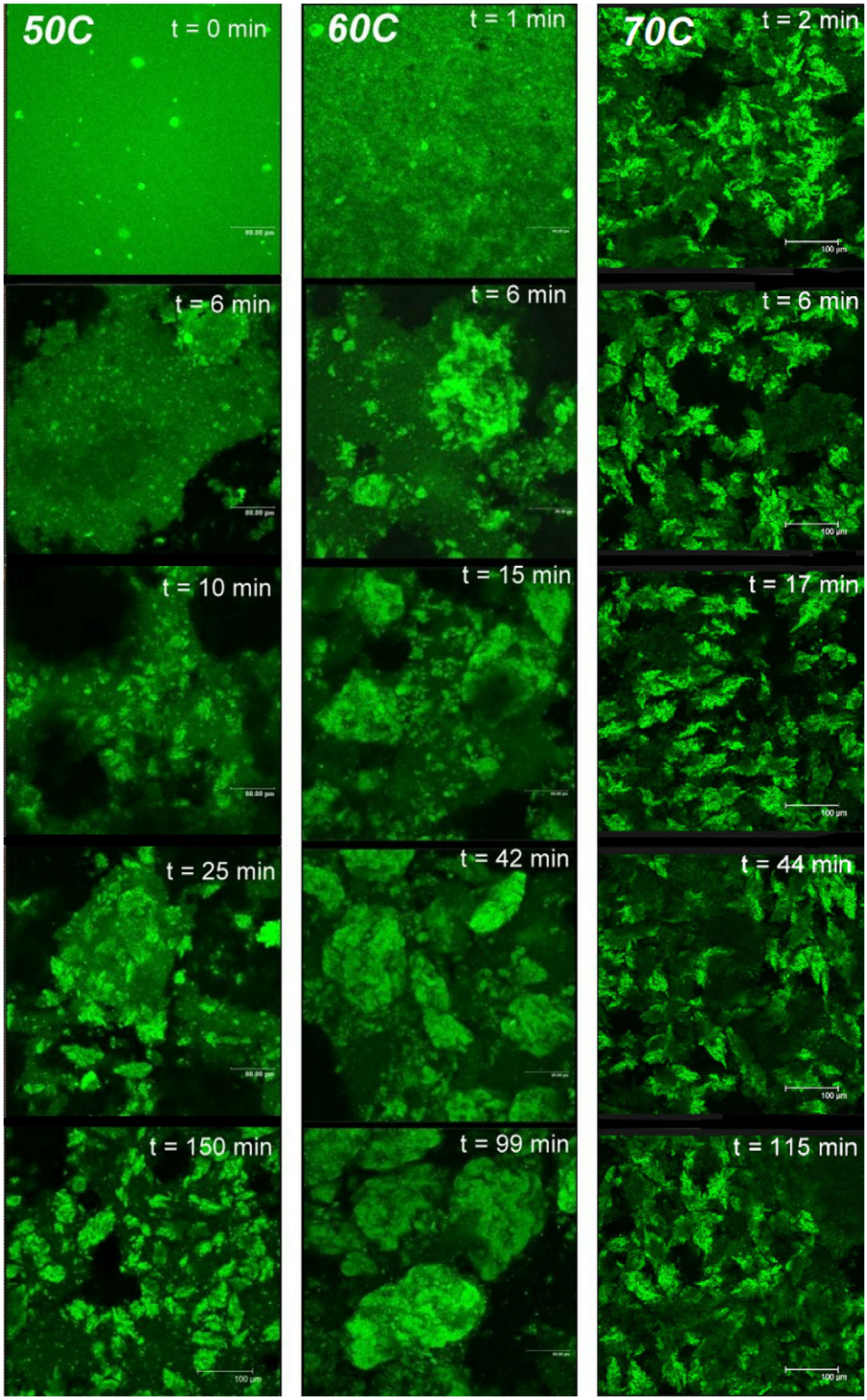

Fig. 9 – Confocal fluorescence microscopy of 10% WPI solutions *vs.* time during aggregation at 50, 60 or 70 °C and pH 4. Lighter areas represent zones of denser protein population. Scale: 100 μm.

for β-LG at pH 4, above which the precipitation rate of β-LG increased significantly with both temperature and time. At 70 °C, β-LG recovery in the alpha fraction increased logarithmically with time, up to 63% after 120 min reaction. Thus, even though α-LA recovery is also maximized, excess precipitation of β-LG reduced the purity of α-LA aggregates to ~45% (Fig. 10C). From 50 to 60 °C, the purity of the alpha fraction increased because α-LA recovery accelerated with temperature, while that of β-LG remained constant. On the other hand, between 65 and 70 °C, α-LA recovery remained constant (maximum), while β-LG precipitation increased with temperature, resulting in a growing proportion of β-LG in the aggregates. Linear fits of these two trends indicate $T \sim 61.5$ °C as the optimal temperature yielding the highest alpha fraction purity. Practically, the range $58 < T < 62$ °C was considered optimal to maximize the rate of α-LA precipitation while minimizing that of β-LG, with a median value of 60 °C.

Table 1 – Effect of WPI concentration on protein compositions and recoveries in the alpha fraction after 120 min at pH 4.6–4.7 (SDS-PAGE data).

	C_A (g/L)	$Y_{agg/wet}$	R_A	R_B	x_A	$R_{B/agg}$ (Eq. (5))	$R_{A/agg}$ (Eq. (6))
28.5 wt.% WPI	80	0.36	0.76	0.31	0.52	~0	0.62
9.8 wt.% WPI	27	0.13	0.50	0.15	0.57	~0	0.42
Dilute WPC[a]	4.1	–	~0.22	~0.01	–	~0	~0.2

[a] From (Bramaud et al., 1997a).

Below 62 °C, longer reaction times also improved enrichment of α-LA in the alpha fraction without increasing β-LG contents, possibly due to the growth of aggregate particles with time enabling better separation during centrifugation.

Aggregate fractions contained ~79–85% (w/w) water and 15–21% solids after centrifugation (from mass difference), indicating that α-LA aggregate particles are loosely packed and loaded with β-LG-rich solution owing to the high water-sorption capacity of α-LA (Kinsella and Fox, 1986; Rantamaki et al., 2000). The main question is whether β-LG recovered within the alpha fraction proceeded solely from entrapment in the water-swollen α-LA particles, or also from β-LG precipitation. Assuming that the β-LG-rich solution was homogeneously distributed throughout the wet sediment, the total recovery of β-LG in the aggregate fraction is equal to the recovery of β-LG *via* precipitation and aggregation, $R_{B/agg}$, plus β-LG contained in the entrapped volume of solution and proportional to the mass fraction of wet aggregates, $Y_{agg/wet}$, as defined in Eq. (4):

$$R_B = R_{B/agg} + (1 - R_{B/agg}) \cdot Y_{agg/wet} \quad (4)$$

then,

$$R_{B/agg} = \frac{(R_B - Y_{agg/wet})}{(1 - Y_{agg/wet})} \quad (5)$$

Calculated values of $R_{B/agg}$ did not vary much with reaction time and averaged 0–3% (w/w) at all values of pH, except at pH 4.3 where $R_{B/agg}$ increased slowly with time, up to 5.6% after 130 min reaction. Thus, $R_{B/agg}$ was considered negligible in most cases, and most of the β-LG recovered in the alpha fraction was attributed to entrapment of solution in the α-LA aggregates by liquid-holding, except near pH 4.3 where a small percentage of β-LG precipitated. This phenomenon was also observed by Bramaud et al. (1997a) at a similar pH of 4.2. We therefore observed no apparent interaction between denatured α-LA and solubilized β-LG between pH 3 and 5.5 at 60 °C. Similarly, the proportion of soluble CMP entrapped in the aggregate fraction *via* liquid-holding and equaled to $Y_{agg/wet}$ constituted 6–9% of the dry precipitate mass. The composition of the aggregate fraction can easily be adjusted accordingly.

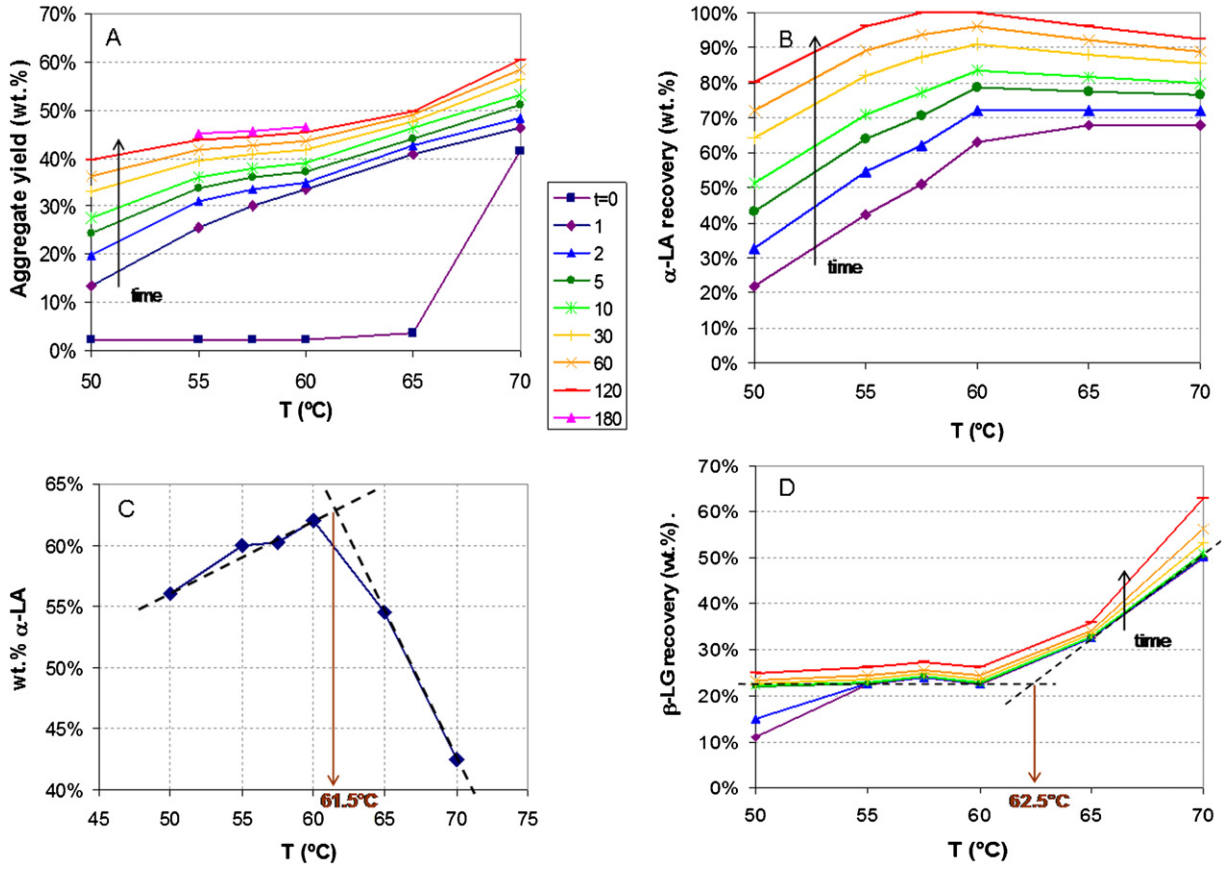

Fig. 10 – Properties of the aggregate fraction as a function of time and temperature during fractionation of 10% WPI solutions at pH 4: (A) total aggregate yield; (B) recovery of α-LA; (C) proportion of α-LA after 120 min; (D) recovery of β-LG.

3.4.3. *Effects of WPI concentration at 60 °C*

At constant operating parameters (pH 4 and 60 °C), reducing the concentration, C_{WPI}, of the WPI solution would produce a smaller volume of wet aggregates and decrease β-LG concentration in the solution, thus considerably reducing the amount of β-LG entrapped in the alpha fraction through liquid holding. Inversely, higher values of C_{WPI} are expected to increase the volume fraction of wet aggregates produced and decrease their purity. However, because the α-LA aggregation reaction is kinetically limited, α-LA precipitation accelerates with increased C_{WPI} and the purity of the alpha fraction does not degrade as quickly as expected from liquid holding. The rate of α-LA recovery due to aggregation only, $R_{A/agg}$, was estimated by:

$$R_{A/agg} = \frac{(R_A - Y_{agg/wet})}{(1 - Y_{agg/wet})} \quad (6)$$

WPI solutions with 9.8 or 28.5% WPI were treated for 120 min at 60 °C and similar pH values of 4.70 and 4.63, respectively. In both cases, $R_{A/agg}$ followed a logarithmic function of time, with: $R_{A/agg} \approx 0.091 \ln(t) + 0.2$ when $C_{WPI} = 28.5\%$; and $R_{A/agg} \approx 0.066 \ln(t) + 0.065$ when $C_{WPI} = 9.8\%$. Tripling the solution's protein concentration almost doubled $R_{A/agg}$, while $R_{B/agg}$ (Eq. (5)) remained approximately null at all times. However, because the mass of wet aggregates, $Y_{agg/wet}$, almost tripled (Table 1), the *total* recovery of β-LG in the precipitate, R_B, increased more than that of α-LA, R_A, and the purity of the alpha fraction decreased slightly, from 0.57 to 0.52.

4. Conclusions

The rate of acid-induced aggregation of α-LA varies greatly with pH, and is also sensitive to both temperature and α-LA concentration. At low pH (<3.8), solution gelling rendered the separation of the fractions difficult. Between pH 3.8–5.5 and 50–70 °C, the precipitation and aggregation of α-LA proteins were considered kinetically limited, and accelerated as pH decreased or temperature increased, while the aggregation of β-LG at 60 °C was negligible between pH 3.8–5.5. At low temperature (≤55 °C), aggregate yield and production rate were low, while higher temperatures (≥65 °C) triggered heavy β-LG denaturation and precipitation. For 10% WPI solutions, the optimal pH and temperature range were defined as pH 4.0–4.1 and T = 60–62 °C, producing both a high aggregate yield (≥40%) and optimal protein fractionation after only 10 min reaction, with α-LA recovery ≥80% in the alpha fraction, and β-LG recovery ≥70% in the beta fraction. Longer reaction times improved these results further: after 60 min at pH 4 and 60 °C, the reaction yielded as much as 46% (w/w) aggregated alpha fraction with 99% α-LA recovery and 58% α-LA purity (27% β-LG, 8% CMP and 7% minor proteins), and a soluble beta fraction with 74% β-LG recovery, and 75.5% β-LG purity (23% CMP and 1.5% α-LA).

Adjusting the concentration of the WPI solution can improve either the products yield or purity, depending on the targeted application: dilute WPI solutions will produce purer alpha fractions, while concentrated solutions (up to 30%) result in higher process efficiency by increasing products yields and reducing the amount of water handled by the process. The purity of the α-LA fraction may also be increased through additional processing steps such as high-speed centrifugation and successive washes to reduce liquid holding and the amount of β-LG entrapped in the aggregates.

Acknowledgments

The authors appreciate the help of Z. Muir and D. VanHekken with gel electrophoresis work, P. Cooke with microscopy imaging, and P. Qi and E. Wickham for assistance.

References

Bernal, V., Jelen, P., 1984. Effect of calcium binding on thermal denaturation of bovine α-lactalbumin. J. Dairy Sci. 67, 2452–2454.

Bonnaillie, L.M., Tomasula, P.M., 2008. Whey protein fractionation. In: Onwulata, C.I., Huth, P.J. (Eds.), Whey Processing, Functionality and Health Benefits. Wiley-Blackwell.

Boye, J.I., Alli, I., 2000. Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a different scanning calorimetric study. Food Res. Int. 33, 673–682.

Bramaud, C., Aimar, P., Daufin, G., 1997a. Optimisation of a whey protein fractionation process based on the selective precipitation of α-lactalbumin. Lait 77, 411–423.

Bramaud, C., Aimar, P., Daufin, G., 1997b. Whey protein fractionation: isoelectric precipitation of α-lactalbumin under gentle heat treatment. Biotechnol. Bioeng. 56, 391–397.

Dannenberg, F., Kessler, H.G., 1988. Reaction kinetics of the denaturation of whey proteins in milk. J. Food Sci. 53, 258–263.

El-Sayed, M.M.H., Chase, H.A., 2011. Trends in whey protein fractionation. Biotechnol. Lett. 33, 1501–1511.

Fernandez, A., Menendez, V., Riera, F.A., Alvarez, R., 2011. Caseinomacropeptide behaviour in a whey protein fractionation process based on alpha-lactalbumin precipitation. J. Dairy Res. 78, 196–202.

Havea, P., Singh, H., Creamer, L.K., 2002. Heat-induced aggregation of whey proteins: comparison of cheese WPC with acid WPC and relevance of mineral composition. J. Agric. Food Chem. 50, 4674–4681.

Ju, Z.Y., Hettiarachchy, N., Kilara, A., 1999. Thermal properties of whey protein aggregates. J. Dairy Sci. 82, 1882–1889.

Kessler, H.G., Beyer, H.J., 1991. Thermal denaturation of whey proteins and its effect in dairy technology. Int. J. Biol. Macromol. 13, 165–173.

Kiesner, C., Clawin-Radecker, I., Meisel, H., Buchheim, W., 2000. Manufacturing of alpha-lactalbumin-enriched whey systems by selective thermal treatment in combination with membrane processes. Lait 80, 99–111.

Kilara, A., Vaghela, M.N., 2004. Whey proteins. In: Yada, R.Y. (Ed.), Proteins in Food Processing. Woodhead Publishing, Cambridge, England, pp. 72–99.

Kinsella, J.E., Fox, P.F., 1986. Water sorption by proteins: milk and whey proteins. Crit. Rev. Food Sci. Nutr. 24, 91–139.

Lamb, H., 1994. Hydrodynamics, 6th edition. Cambridge University Press.

McKenzie, H.A., 1971. Milk Proteins. Chemistry and Molecular Biology. New York, Academic Press.

Outinen, M., Tossavainen, O., Tupasela, T., Koskela, P., Koskinen, H., Rantamäki, P., Syväoja, E.-L., Antila, P., Kankare, V., 1996. Fractionation of proteins from whey with different pilot scale processes. LWT - Food Sci. Technol. 29, 411–417.

Pearce, R.J., 1983. Thermal separation of β-lactoglobulin and α-lactalbumin in bovine cheddar cheese whey. Aust. J. Dairy Technol. 38, 144–149.

Pearce, R.J., Dunkerley, J.A., Marshall, S.C., 1991a. New dairy science and technology leads to novel milk protein products. Food Res. Quart. 51, 145–157.

Permyakov, E.A., Berliner, L.J., 2000. α-Lactalbumin: structure and function. FEBS Lett. 473, 269–274.

Plock, J., Spiegel, T., Kessler, H.G., 1998. Influence of the lactose concentration on the denaturation kinetics of whey proteins in concentrated sweet whey. Milchwissenschaft-Milk Sci. Int. 53, 389–393.

Rantamaki, P., Tossavainen, O., Outinen, M., Tupasela, T., Koskela, P., Kaunismaki, M., 2000. Functional properties of the whey protein fractions produced in pilot scale processes. Foaming,

water-holding capacity and gelation. Milchwissenschaft-Milk Sci. Int. 55, 569–572.
Rialland, J.-P., Barbier, J.-P., 1988. Process for selectively separating the α-lactalbumin from the proteins of whey. U.S. Patent no. 4782138.
Roefs, S.P.F.M., de Kruif, C.G., 1994. A model for the denaturation and aggregation of β-lactoglobulin. Eur. J. Biochem. 226, 883–889.
Schmitt, C., Bovay, C., Rouvet, M., Shojaei-Rami, S., Kolodziejczyk, E., 2007. Whey protein soluble aggregates from heating with NaCl: physicochemical, interfacial, and foaming properties. Langmuir 23, 4155–4166.
Spiegel, T., Huss, M., 2002. Whey protein aggregation under shear conditions – effects of pH-value and removal of calcium. Int. J. Food Sci. Technol. 37, 559–568.
Tolkach, A., Kulozik, U., 2005. Effect of pH and temperature on the reaction kinetic parameters of the thermal denaturation of β-lactoglobulin. Milchwissenschaft-Milk Sci. Int. 60, 248–252.
Tolkach, A., Steinle, S., Kulozik, U., 2005. Optimization of thermal pretreatment conditions for the separation of native α-lactalbumin from whey protein concentrates by means of selective denaturation of β-lactoglobulin. J. Food Sci. 70, E557–E566.
Tomasula, P.M., Parris, N., 1999. Whey protein fractionation using high pressure or supercritical carbon dioxide. U.S. Patent no. 5,925,737.
Tomasula, P.M., Parris, N., Boswell, R.T., Moten, R.O., 1998. Preparation of enriched fractions of α-lactalbumin and β-lactoglobulin from cheese whey using carbon dioxide. J. Food Proc. Preserv. 22, 463–476.
Tomasula, P.M., Yee, W.C.F., 2001. Enriched fractions of alpha-lactalbumin (α-La) and beta-lactoglobulin (β-LG) from whey protein concentrate using carbon dioxide. Functional properties in aqueous solution. J. Food Proc. Preserv. 25, 267–282.
Verheul, M., Roefs, S.P.F.M., de Kruif, K.G., 1998. Kinetics of heat-induced aggregation of β-lactoglobulin. J. Agric. Food Chem. 46, 896–903.
Vivian, J.T., Callis, P.R., 2001. Mechanisms of tryptophan fluorescence shifts in proteins. Biophys. J. 80, 2093–2109.
Wang, Q., Tolkach, A., Kulozik, U., 2006. Quantitative assessment of thermal denaturation of bovine α-lactalbumin via low-intensity ultrasound, HPLC, and DSC. J. Agric. Food Chem. 54, 6501–6506.
Wu, C., 2003. Whey treatment process for achieving high concentration of α-lactalbumin. U.S. Patent.
Wu, H., Xie, J.J., Morbidelli, M., 2005. Kinetics of cold-set diffusion-limited aggregations of denatured whey protein isolate colloids. Biomacromolecules 6, 3189–3197.